Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 2375–2379, March 1993
Genetics

Disruption of the transthyretin gene results in mice with depressed levels of plasma retinol and thyroid hormone

(homologous recombination/retinol-binding protein)

VASSO EPISKOPOU*†, SHUICHIRO MAEDA‡, SEIJI NISHIGUCHI§, KAZUNORI SHIMADA§, GEORGE A. GAITANARIS¶, MAX E. GOTTESMAN¶, AND ELIZABETH J. ROBERTSON*,||,**

*Department of Genetics & Development and ¶Institute of Cancer Research, Columbia University College of Physicians & Surgeons, 701 West 168th Street, New York, NY 10032; †Department of Biochemistry and Molecular Genetics, St. Mary's Hospital, Medical School, Norfolk Place, London W2 1PG, United Kingdom; ‡Department of Biochemistry, Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto 860, Japan; §Department of Medical Genetics, Division of Molecular Biomedicine, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565, Japan; and ||The Biological Laboratories, Department of Cellular & Developmental Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138

Communicated by I. S. Edelman, December 10, 1992

ABSTRACT **Transthyretin (TTR) is thought to play a major role in vitamin A metabolism and thyroid hormone transport in mammals. To investigate the physiological role of the TTR protein in development of the embryo and in the adult, we used gene targeting techniques to generate a null mutation at the mouse *ttr* locus. The resultant mutant animals are phenotypically normal, viable, and fertile. However, levels of serum retinol, retinol-binding protein, and thyroid hormone are significantly depressed in the mutant animals. These observations demonstrate that the TTR protein maintains normal levels of these metabolites in the circulating plasma.**

Transthyretin (TTR) is a 55-kDa plasma protein consisting of four identical subunits of 127 amino acids each (1, 2). The mouse TTR gene has been cloned and shows 80% homology with the human gene at the protein level (3). In adults, TTR is synthesized at high levels in the liver and choroid plexus (4–7). TTR is secreted into the plasma from the liver and into the cerebrospinal fluid by the choroid plexus. TTR is thought to have at least two functions in the adult mammal. (*i*) TTR possesses high-affinity binding sites for the thyroid hormones T_3 and T_4 (thyroxin) and is the major thyroid hormone carrier in rodent plasma (8). (*ii*) TTR forms a macromolecular complex with retinol-binding protein (RBP) in association with retinol, which may prevent filtration of the plasma RBP–retinol complex through the kidney (9, 10).

In situ hybridization studies have documented high levels of expression of the *ttr* and *rbp* genes in the visceral endoderm tissue of the early post-implantation rodent embryo (11). Thus, it seems likely that these molecules are required for the transport of retinol from the maternal blood to the fetus. By late gestation, synthesis of TTR, like that of the adult, is confined primarily to the liver and the epithelial cell layer of the choroid plexus (12, 13). Since vitamin A (retinol) is required for normal development (14), these expression studies implied that TTR functioned in the developing embryo and the developing central nervous system to transport retinol and/or thyroid hormone.

A genetic disease associated with TTR has been extensively studied. TTR variants with single amino acid substitutions are the main cause of an autosomal and dominantly inherited disease, familial amyloidotic polyneuropathy in humans (15–19).

To examine the developmental and metabolic roles of TTR, we have generated a mutant mouse strain carrying a null mutation at the *ttr* locus. Mice homozygous for the mutated gene appear normal and fertile, although they have no detectable plasma retinol (lower limit of detection, 3%), and have depressed levels of thyroid hormone.

MATERIALS AND METHODS

Construction of the Targeting Vector. The targeting vector contains a 5.9-kb *Avr* II–*Sca* I genomic fragment, including exons 1–3, the *neo* and the herpes simplex virus thymidine kinase (*tk*) genes. Specifically, the 1.1-kb blunt-ended *Xho* I–*Hin*dIII fragment from PMC1*NEO*polyA [Stratagene; a derivative of PMC1*neo* containing a poly(A) signal] was inserted into the *Bam*HI site of the second *ttr* exon. The 1.85-kb *Xho* I–*Sal* I fragment of PMC1*tk* (20) was added to the 3′ end of the vector.

Cell Culture. CCE embryonic stem (ES) cells (21) were maintained on STO feeder layers as described (22). Cells were electroporated and selected as described (23). Individual drug-resistant colonies were picked and expanded for analysis.

DNA Analysis. The PCR analysis was performed with Perkin–Elmer/Cetus kit and cycler. The DNA from the ES cell clones was purified with phenol, chloroform, and ethanol precipitation. The oligomers used for the specific amplification were 5′ end primer 1 (5′-GAGCGAGTGTTCCGATACTCTAA-3′), which corresponds to 181 bp upstream from the presumed transcription initiation site of the mouse *ttr* gene (3), and 3′ end primer 2 (5′-GCGCTGACAGCCGGAACACG-3′), which corresponds to 413 bp downstream from the beginning of the *neo* cassette. The annealing temperature was 64°C.

Embryo Manipulations. Approximately 15 cells were injected into MF1 (Harlan–Sprague–Dawley) host blastocysts collected 3.5 days postcoitus, as described (24). Chimerism was scored by coat and eye pigmentation in the MF1 albino background.

Immunoblot. Four-microliter samples of serum (diluted 1:10) were analyzed using a rabbit anti-rat TTR antibody (25, 26). 15% SDS/PAGE was performed as described (27), and the separated proteins were transferred to an Immobilon-P membrane (Millipore). Detection of specific TTR antigens was performed with the polyclonal anti-rat TTR antibody and goat peroxidase-labeled anti-rabbit IgG (Boehringer Mannheim) as the second antibody.

Histopathological and Plasma Analysis. Four homozygous, two heterozygous, and two wild-type siblings were subject to a complete histopathological analysis (Cenvet, Woodside, NY) that included examination of sciatic nerve and organs

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: TTR, transthyretin; RBP, retinol-binding protein; ES, embryonic stem.

**To whom reprint requests should be addressed.

that normally express *ttr* gene: brain, kidney, and liver (3–7). No evidence of abnormalities was found in any group. Total T_4 and T_3 levels were determined using canine double antibody and Coat-A-Count canine radioimmunoassay (RIA) (Diagnostic Products, Los Angeles). Retinol level was determined by HPLC (28) and RBP levels were determined by RIA as described (29).

Diet Experiment and Mouse Breeding. Mating pairs of homozygous, heterozygous, and wild-type animals were maintained on a diet of vitamin A-depleted lab chow (Test Diet, Purina) and housed on autoclaved bedding (to destroy vitamin A). Animals were inspected at 2-day intervals for symptoms of vitamin A deprivation over several months. All groups, irrespective of genotype, proved to be fertile. F_1 progeny obtained from all groups of experimental animals developed signs of vitamin A deprivation.

RESULTS AND DISCUSSION

***ttr* Gene Targeting.** The mouse *ttr* gene was disrupted using the technique of gene targeting in ES cells (30, 31). As *ttr* is not expressed in ES cells, we used a positive negative selection strategy (20). The targeting vector was a replacement vector containing two selectable markers (Fig. 1), the bacterial neomycin-resistance gene (*neo*) for positive selection and the herpes simplex virus thymidine kinase gene (*tk*) for negative selection. The MC1*neo* expression cassette (32) was introduced into the second exon of a 5.9-kb genomic mouse *ttr* gene fragment that carries exons 1–3 (3). The MC1*tk* cassette was added at the 3′ end of the *ttr* gene.

Following transfection into the CCE ES cell line, targeted events were detected by PCR amplification of a 1.8-kb junction DNA fragment generated by homologous recombination (Fig. 1). DNA from six independent colonies yielded a product of the predicted size, which, as expected, hybridized to a *ttr-neo* probe (Fig. 1). The presence of the *ttr* mutation in the six candidate ES cell clones was confirmed by restriction analysis of genomic DNA (Fig. 1). The overall targeting frequency obtained with this vector was ≈1 per 80 integration events.

TTR-Deficient Mice. After injection of the ES cell clones into MF1 host blastocysts two germ-line chimeras were obtained. These animals were bred with MF1 females and transmitted the disrupted *ttr* allele to 50% of their progeny.

Next, heterozygous animals were intercrossed. Genotyping of the resulting progeny of 50 different litters with an average litter size of 8 showed that live-born mice homozygous for the disrupted *ttr* gene (referred to hereafter as "homozygotes") were recovered at the predicted frequency (Fig. 2), indicating that absence of TTR does not compromise fetal development. Homozygous animals display no obvious phenotypic abnormalities postnatally, as determined morphologically and by histopathological analysis. In addition, their longevity does not differ from their heterozygous or wild-type siblings. Breeding experiments have subsequently established that the fertility of homozygous mice of both sexes is normal.

Confirmation of a *ttr* Null Allele. To verify that the gene targeting event had in fact generated a null mutation at the *ttr* locus, we examined TTR levels in peripheral blood of homozygotes by Western blot analysis. We used a rat TTR-specific polyclonal antiserum (25, 26) that cross-reacts with mouse TTR (the two proteins differ in only 7 amino acid residues; refs. 33 and 34). We observed that none of the homozygotes had detectable plasma TTR, nor were truncated forms of TTR detected (Fig. 3). Moreover, as pre-

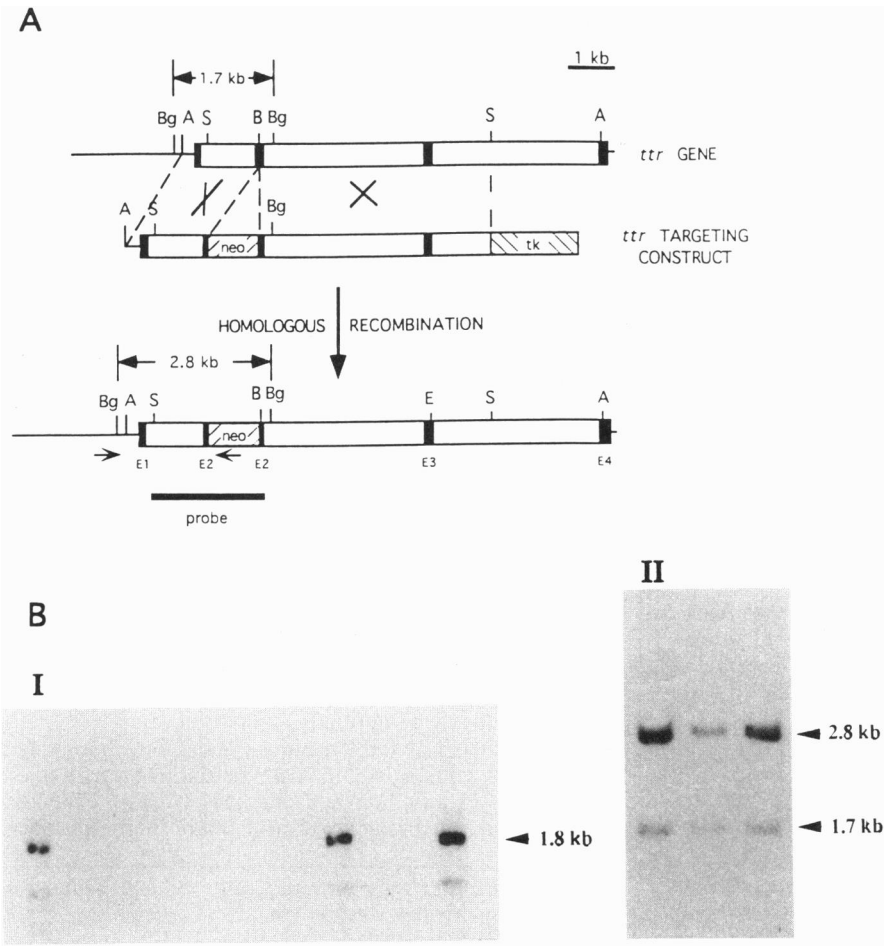

FIG. 1. Targeted disruption of the *ttr* gene in ES cells. (*A*) Restriction maps of the mouse *ttr* genomic locus (2), the replacement vector, and the predicted structure of the targeted *ttr* gene. Filled boxes represent exons (E1–E4), open boxes indicate introns, and bars denote flanking regions of the *ttr* locus. The small arrows indicate the position of the oligonucleotide primers that were used for PCR assays (30). For Southern blot hybridization the probe used was the *Sca* I–*Bam*HI fragment (probe A). A, *Avr* II; B, *Bam*HI; Bg, *Bgl* II; E, *Eco*RI; S, *Sca* I. (*B*) Southern blot analysis of DNA of targeted clones. I. PCR amplified DNAs from G418-resistant and GANC-resistant individual colonies were hybridized to probe A. A 1.8-kb fragment is expected from clones carrying a disrupted *ttr* gene. II. Southern blot analysis of DNA from targeted ES cell clones. Hybridization of the *Bgl* II-digested genomic DNA with probe A should give two bands: 1.7 kb from the wild-type allele and 2.8 kb corresponding to the mutant allele (due to insertion of the 1.1-kb MC1*neo* sequences at the *ttr* locus).

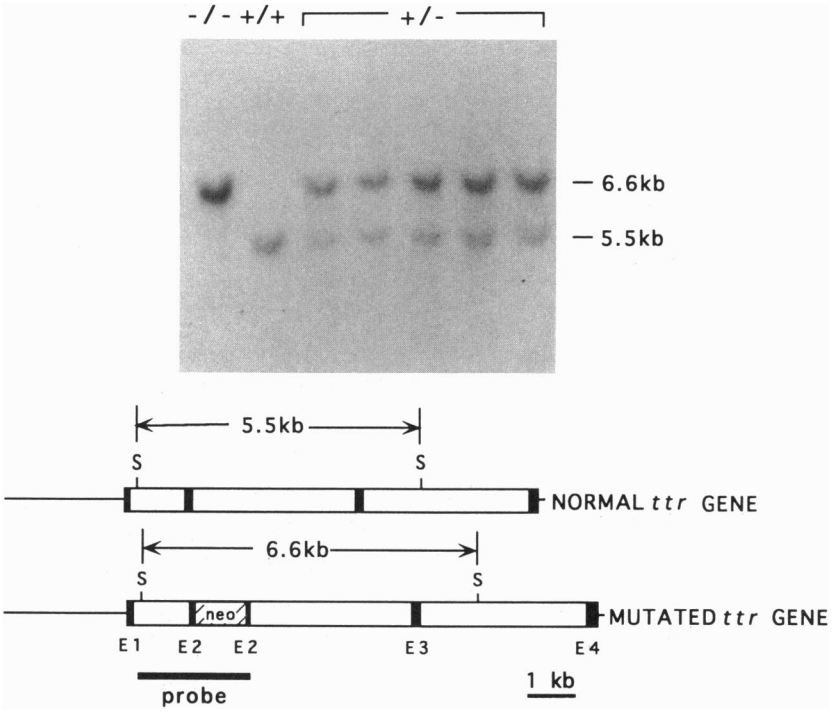

FIG. 2. Southern blot analysis of tail DNA samples from live offsprings obtained from intercrossing animals heterozygous for the *ttr* mutation. Probe A (Fig. 1) was used to hybridize a Southern blot of *Sca* I-digested DNA from individual offspring. The hybridizing bands are predicted to be 5.5 kb for the wild-type allele and 6.6 kb for the disrupted allele. The genotype of animals is indicated as +/+ (wild type), +/− (heterozygous), and −/− (homozygous).

dicted, the heterozygous animals have intermediate levels of plasma TTR. Metabolic labeling experiments using choroid plexus tissue confirmed that no TTR protein, or altered forms of the protein, was expressed in the homozygous mice (J. Palha and M. J. Saraiva, personal communication). Thus we can conclude that TTR protein is not essential for embryonic development, postnatal viability, or fertility.

Plasma Levels of the Thyroid Hormone. Thyroid hormones are iodinated derivatives of the amino acid tyrosine. Thyroxin (T_4) contains four iodine atoms and is synthesized exclusively in the thyroid gland. Triiodothyronine (T_3) is derived from T_4 by a 5′-deiodinase, a microsomal enzyme that is especially prominent in liver and kidney (35). T_3 has a significantly shorter half-life than T_4 but has more metabolic activity than thyroxin. T_4 and T_3 circulate bound to TTR and other plasma proteins (8, 36).

In humans, TTR is not the major carrier of T_4 in the plasma, although in the cerebrospinal fluid, 80% of T_4 is bound to TTR (37). In the adult mouse, however, TTR is the major plasma carrier of T_4 (8). It was therefore of interest to measure the levels of circulating thyroid hormones in these TTR-deficient animals. We found that the total T_4 level was reduced almost 3-fold relative to age-matched controls (Table 1). In contrast, the level of total T_3 in the plasma of the homozygotes was 65% of control values (Table 1). Binding to albumin may account for the retention of plasma T_3 in the homozygous mice. The T_3 and T_4 levels lie between the homozygotes and the wild-type values in the heterozygotes, reflecting the decreased levels of circulating TTR in the plasma of the heterozygotes (J. Palha and M. J. M. Saraiva, personal communication).

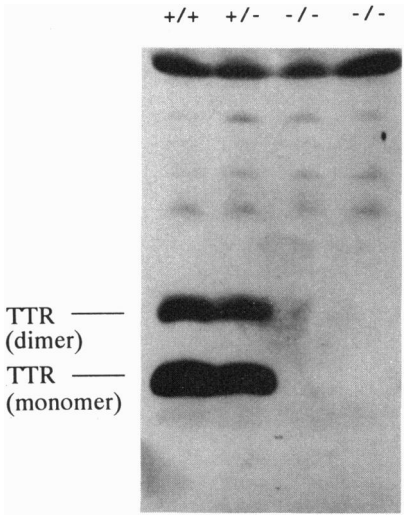

FIG. 3. Western blot analysis of serum samples from animals that were wild type (+/+), heterozygous (+/−), or homozygous (−/−) for the targeted ttr allele.

These results are of interest, since, under physiological conditions, conversion of T_4 to T_3 is thought to be proportional to the concentration of the T_4 substrate. The possibility that homozygous mice have compensated for the lowered T_4 levels by increasing 5′-deiodinase activity and/or enhancing the uptake of T_4 in the liver and kidney must be investigated.

Thyroid hormone production is regulated by pituitary thyrotropin (TSH). Circulating T_3 and T_4 levels have inhibitory effects on the synthesis and release of TSH (38). In view of the depressed levels of plasma T_4 in the homozygotes, it seemed reasonable to expect a perturbation in plasma TSH levels. Instead, we found no indications that TSH levels are affected in the TTR-deficient mice (J. Palha and M. J. M. Saraiva, personal communication). Furthermore, the thyroid displays no gross morphological abnormalities in the homozygous mice (data not shown). We assume that the homozygous mice are euthyroid because they have only slightly reduced T_3 levels. Interestingly, humans deficient in thyroxin-binding globulin, which is the major thyroid hor-

Table 1. Plasma levels of total T_4 and T_3

Genotype	*n*	Total T_4, μg/dl	Total T_3, ng/dl
−/−	9	1.9 ± 0.6	72 ± 21
+/+	8	5.5 ± 1.0	112 ± 30

The values given for total T_4 and T_3 represent means ± SD. The *n* values refer to number of mice used.

Table 2. Plasma levels of retinol and RBP

Genotype	*n*	Retinol, μg/dl	RBP, mg/dl
−/−	6	<2.0	0.11 ± 0.08
+/+	5	30.0 ± 1.2	3.41 ± 1.21

The values given for retinol and RBP represent means ± SD. The *n* values refer to number of mice used.

mone carrier in humans, have depressed T_4 levels and are euthyroid (39).

Plasma Levels of Retinol and RBP. In mammals, a well-regulated transport and storage system provides tissues with the correct amounts of retinoids in spite of normal fluctuations in daily vitamin A intake. Retinyl esters obtained from the diet are delivered to the liver and this retinoid is either stored or secreted as retinol bound to RBP into the plasma. Most of the retinol–RBP in the plasma is reversibly complexed with TTR and it has been proposed that the retinol–RBP–TTR complex is less susceptible to filtration by the kidney glomeruli (9–10).

Consistent with the hypothesis that TTR prevents loss of RBP–retinol, we find that the plasma level of retinol is below the level of detection (<6% of the normal value) and the level of RBP is 3% of the normal value (Table 2). Although these data strongly suggest that TTR plays a direct role in the plasma transport of the RBP–retinol complex, they do not exclude the possibility that TTR may be also required for RBP secretion from the liver (40).

Although it is clear that the TTR-deficient mice have deficient retinol transport, these mice do not show any symptoms of vitamin A deficiency. Preliminary results show that the liver levels of RBP and retinol in the homozygotes are not depressed (S. Wei and W. S. Blaner, personal communication). Since the overall supply of stored retinol in these animals appears to be adequate, the TTR-deficient mice are able to recover normal amounts of vitamin A from their diet.

After uptake by intestinal cells, dietary retinoids are delivered primarily to the liver by chylomicron remnants in the form of retinyl esters (41–44). Extrahepatic uptake of chylomicron remnants has been reported to occur in several tissues (41–45) and it is known that retinoic acid (RA) can also be absorbed through the portal vein directly from the diet and transported bound to albumin (9–10). We considered the possibility that the homozygotes met their tissue retinol requirements through the daily dietary supply of retinyl esters and RA rather than through retinol–RBP liver depots. To test this hypothesis, homozygous, heterozygous, and wild-type mice were subjected to a totally retinoid-deficient diet as weanlings and were maintained on this diet for several months. If the model were correct, the TTR-deficient animals would develop symptoms of vitamin A deprivation earlier than the heterozygotes and the wild-type controls. In contrast, all animals developed symptoms in the F_1 generation, with similar times of onset. To verify that these symptoms (loss of weight, infections, eye abnormalities, etc.) were due to vitamin A deficiency, we returned the survivors to a control vitamin A-sufficient diet. The affected animals recovered within 1 week. We concluded, therefore, that the homozygotes can utilize stored retinol despite a defective plasma retinol transport system.

In summary, mice lacking the *ttr* gene show defective plasma T_4 and retinol transport. These results strongly support the previous data suggesting that TTR plays an important role in the transport of these metabolites. Since TTR-deficient mice are phenotypically normal, a compensatory mechanism(s) may exist that enables them to adapt to the depressed level of T_4 and retinol in the plasma. Alternatively, the depressed levels of T_4 and retinol might be adequate for the daily requirement in the laboratory mouse.

Finally, the TTR-deficient mice may also be used as recipients of the human variant *ttr* genes that are associated with familial amyloidotic polyneuropathy (46). These transgenic mice will be useful for studying the function of the TTR variants and their role in the pathogenesis of the disease.

We thank Joana Palha and Maria Joao Saraiva for performing the thyroid hormone RIAs, Shuanghong Wei, Roseann Piantedosi, and William Blaner for performing the retinol and RBP assays, and Claire Shean for help with the Western blot analysis. This work was supported by Public Health Service Grant RO1 HD 25335 from Child Health and Human Development to E.J.R., by Public Health Service Grant PO1 CA 23767-15 to M.E.G., and by a grant under the Monbusho International Scientific Research Program. V.E. is the recipient of a National Institutes of Health postdoctoral fellowship. E.J.R. is the recipient of a fellowship from the David and Lucile Packard Foundation.

1. Kanda, Y., Goodman, D. S., Canfield, R. E. & Morgan, F. J. (1974) *J. Biol. Chem.* **249**, 6796–6805.
2. Blake, C. F., Geisow, M. J., Oatley, S. J., Rerat, B. & Rerat, C. (1978) *J. Mol. Biol.* **121**, 339–356.
3. Wakasugi, S., Maeda, S. & Shimada, K. (1986) *J. Biochem. (Tokyo)* **100**, 49–58.
4. Dickson, P. W., Howlett, G. J. & Schreiber, G. (1985) *J. Biol. Chem.* **260**, 8214–8219.
5. Martone, R. L., Herbert, J., Dwork, A. & Schon, E. A. (1988) *Biochem. Biophys. Res. Commun.* **151**, 905–912.
6. Felding, P. & Fex, G. (1982) *Biochim. Biophys. Acta* **716**, 446–449.
7. Soprano, D. R., Herbert, J., Soprano, K. J., Schon, E. A. & Goodman, D. S. (1985) *J. Biol. Chem.* **260**, 11793–11798.
8. Vranckx, R., Saru, L., Maya, M. & Nunez, E. (1990) *Biochem. J.* **271**, 373–379.
9. Wolf, G. (1984) *Physiol. Rev.* **64**, 873–937.
10. Goodman, D. S. & Blaner, W. S. (1984) in *The Retinoids*, eds. Sporn, M. B., Roberts, A. B. & Goodman, D. S. (Academic, Orlando, FL), Vol. 2, pp. 1–39.
11. Makover, A., Soprano, D. R., Wyatt, M. L. & Goodman, D. S. (1989) *Differentiation* **40**, 17–25.
12. Soprano, D. R., Soprano, K. J. & Goodman, D. S. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7330–7334.
13. Murakami, T., Yasuda, Y., Mita, S., Maeda, S., Shimada, K., Fujimoto, T. & Araki, S. (1987) *Cell Differ.* **22**, 1–10.
14. Blomhoff, R., Green, M. H., Berg, T. & Norum, K. R. (1990) *Science* **250**, 399–404.
15. Tawara, S., Nakazato, M., Kangawa, K., Matsuo, H. & Araki, S. (1983) *Biochem. Biophys. Res. Commun.* **116**, 880–888.
16. Saraiva, M. J. M., Birken, S., Costa, P. P. & Goodman, D. S. (1984) *J. Clin. Invest.* **74**, 104–119.
17. Dwulet, F. E. & Benson, M. D. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 694–698.
18. Ide, M., Mita, S., Ikegawa, S., Maeda, S., Shimada, K. & Araki, S. (1986) *Hum. Genet.* **73**, 281–285.
19. Benson, M. D. (1988) *Trends NeuroSci.* **12**, 88–92.
20. Mansour, S. L., Thomas, K. R. & Capecchi, M. R. (1988) *Nature (London)* **336**, 348–352.
21. Robertson, E., Bradley, A., Kuehn, M. & Evans, M. (1986) *Nature (London)* **323**, 445–448.
22. Robertson, E. J. (1987) in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, ed. Robertson, E. J. (IRL, Oxford), pp. 71–112.
23. DeChiara, T. M., Efstratiadis, A. & Robertson, E. J. (1990) *Nature (London)* **345**, 78–80.
24. Bradley, A. (1987) in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, ed. Robertson, E. J. (IRL, Oxford), pp. 113–151.
25. Navab, M., Mallia, A. K., Kanda, Y. & Goodman, D. S. (1977) *J. Biol. Chem.* **252**, 5100–5106.
26. Brouwer, A., Blaner, W. S., Kukler, A. & van den Berg, K. J. (1988) *Chem-Biol. Interact.* **68**, 203–217.
27. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
28. Freidman, G. D., Blaner, W. S., Goodman, D. S., Vogelman, J. H., Brind, J. L., Hoover, R., Fireman, B. H. & Orentreich, N. O. (1986) *Am. J. Epidemiol.* **123**, 781–789.
29. Blaner, W. S. (1990) *Methods Enzymol.* **189**, 270–281.

30. Capecchi, M. R. (1989) *Science* **244,** 1288–1292.
31. Robertson, E. J. (1991) *Biol. Reprod.* **44,** 238–245.
32. Thomas, K. R. & Capecchi, M. R. (1987) *Cell* **51,** 503–512.
33. Sundelin, J., Melhus, H., Das, S., Eriksson, U., Lind, P., Tragardh, L., Peterson, P. A. & Rask, L. (1985) *J. Biol. Chem.* **260,** 6481–6487.
34. Wakasugi, S., Maeda, S., Shimada, K., Nakashima, H. & Migita, S. (1985) *J. Biochem. (Tokyo)* **98,** 1707–1714.
35. Berry, M. J., Banu, L. & Larsen, P. R. (1991) *Nature (London)* **349,** 438–440.
36. Pages, R. A., Robbins, J. & Edelhoch, H. (1973) *Biochemistry* **12,** 2773–2779.
37. Herbert, J., Wilcox, J., Pham, K. T., Fremeau, R. T., Zeviani, M., Dwork, A., Soprano, D. R., Makover, A., Goodman-Dewitt, S., Zimmerman, E. A., Roberts, J. L. & Schon, E. A. (1986) *Neurology* **36,** 900–911.
38. Larsen, P. R. (1982) *N. Engl. J. Med.* **306,** 23–32.
39. Burr, W. A., Ramsden, D. B. & Hoffenberg, R. (1980) *Q. J. Med.* **49,** 295–313.
40. Melhus, H., Nilsson, T., Peterson, P. A. & Rask, L. (1991) *Exp. Cell Res.* **197,** 119–124.
41. Green, P. H. R. & Glickman, R. M. (1981) *J. Lipid Res.* **22,** 1153–1173.
42. Goodman, D. S., Huang, H. S. & Shiratori, T. (1965) *J. Lipid Res.* **6,** 390–396.
43. Blomhoff, R., Helgerud, P., Ramussen, M., Berg, T. & Norum, K. R. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7326–7330.
44. Blomhoff, R., Holte, K., Naess, L. & Berg, T. (1984) *Exp. Cell Res.* **150,** 186–193.
45. Hussain, M. M., Mahley, R. W., Boyles, J. K., Lindquis, P. A. & Brecht, W. J. (1989) *J. Biol. Chem.* **264,** 17931–17938.
46. Shimada, K., Maeda, S., Murakami, T., Nishiguchi, S., Tashiro, F., Wakasugi, S., Yi, S., Takahashi, K. & Yamamura, K. (1989) *Mol. Biol. Med.* **6,** 333–343.